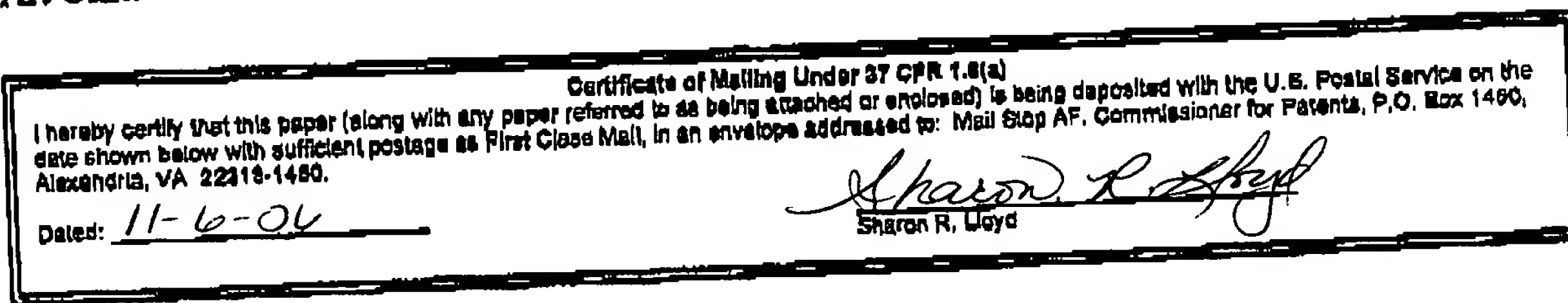




Docket No.: W0571.70010US02
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Thomas Tuschl et al.
Serial No.: 09/821,832
Confirmation No.: 6420
Filed: March 30, 2001
For: RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA
INTERFERENCE
Examiner: Wollenberger, Louis V.
Art Unit: 1635



DECLARATION OF PHILLIP ZAMORE UNDER 37 C.F.R. §1.131

I, Phillip Zamore, state and declare the following:

1. I am a co-inventor of the above-identified patent application with Thomas Tuschl, David Bartel, and Phillip Sharp.
2. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was invented prior to March 16, 2000.
3. Thomas Tuschl, David Bartel, Phillip Sharp and I co-authored a manuscript which was published as Zamore et al Cell 101, 25-33 (2000) on March 31, 2000 (referred to herein as Zamore et al manuscript).
4. The Zamore et al manuscript was accepted for publication by the journal Cell on March 10, 2000. The data described in the manuscript was not revised after March 10, 2000. A copy of the Zamore et al manuscript as it was accepted (published) is attached hereto as Exhibit A.
5. The Zamore et al manuscript included in Exhibit A accurately describes the invention we made prior to March 16, 2000 and provides support for currently pending claims in the above-identified application.
6. Hammond et al was published on March 16, 2000, after the date of acceptance of the Zamore et al manuscript.

NOT AVAILABLE COPY

USSN 09/821,832

2

Art Unit: 1635

7. Accordingly, this Declaration establishes a date of invention for the above-identified application at least as early as March 10, 2000.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this document and any patent which may issue from the above-identified patent application.


Dr. Phillip Zamore

6 November 2006
Date

BEST AVAILABLE COPY

RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals

Phillip D. Zamore,*# Thomas Tuschl,†#
Phillip A. Sharp,‡§ and David P. Bartel§||

*Department of Biochemistry and Molecular Biology
University of Massachusetts Medical School
Worcester, Massachusetts 01655

†Department of Cellular Biochemistry
Max-Planck-Institute for Biophysical Chemistry
Am Faßberg 11

D-37077 Göttingen

Germany

‡Center for Cancer Research and

§Department of Biology

Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

||The Whitehead Institute for Biomedical Research

9 Cambridge Center

Cambridge, Massachusetts 02142

Summary

Double-stranded RNA (dsRNA) directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). Using a recently developed *Drosophila* in vitro system, we examined the molecular mechanism underlying RNAi. We find that RNAi is ATP dependent yet uncoupled from mRNA translation. During the RNAi reaction, both strands of the dsRNA are processed to RNA segments 21–23 nucleotides in length. Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21–23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21–23 nucleotide fragments from the dsRNA are guiding mRNA cleavage.

Introduction

The term RNA interference, or "RNAi," was initially coined by Fire and coworkers (Fire et al., 1998) to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (for reviews see Fire, 1999; Hunter, 2000; Hunter, 1999; Montgomery and Fire, 1998; Sharp, 1999; Wagner and Sun, 1998). Their discovery built upon the previous, puzzling observation that sense and antisense RNA (asRNA) were equally effective in suppressing specific gene expression (Guo and Kemphues, 1995), a paradox resolved by the finding that small amounts of dsRNA contaminate sense and antisense preparations (Fire et al., 1998). RNAi has since been discovered in a wide variety of animals, including flies (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), trypanosomes (Ngo et al., 1998), planaria (Sánchez-Alvarado and Newmark,

1999), hydra (Lohmann et al., 1999), zebrafish (Wargelius et al., 1999), and mice (Wianny and Zernicka-Goetz, 2000), and appears to be related to gene silencing phenomena in plants ("cosuppression"; Vaucheret et al., 1998; Waterhouse et al., 1998, 1999; Baulcombe, 1999) and the fungus *Neurospora* ("quelling"; Cogoni et al., 1996; Cogoni and Macino, 1999a, 1999b).

RNAi occurs posttranscriptionally and involves mRNA degradation (Montgomery et al., 1998; Ngo et al., 1998). In addition to providing a powerful tool for creating gene-specific phenocopies of loss-of-function mutations, RNAi may also play an important biological role in protecting the genome against instability caused by the accumulation of transposons and repetitive sequences (Ketting et al., 1999; Tabara et al., 1999). In *C. elegans*, dsRNA blocks specific gene expression even when expressed by bacteria fed to the worms (Timmons and Fire, 1998). RNAi in animals may also represent an ancient antiviral response, just as posttranscriptional gene silencing appears to protect plants from viral infection (Baulcombe, 1999; Grant, 1999; Ratcliff et al., 1999). The breadth of RNAi-like processes suggests that RNAi may encompass gene silencing phenomena, including cellular strategies for gene regulation, well beyond the initial observation that dsRNA can produce RNAi.

Genetic screens in both *C. elegans* and *Neurospora* have identified genes required for RNAi (Cogoni and Macino, 1997; Tabara et al., 1999). Mutations in a subset of these genes, including *rde-2*, *rde-3*, *mut-2*, and *mut-7*, permit the mobilization of transposons in the worm germline (Ketting et al., 1999; Tabara et al., 1999; Grishok et al., 2000). A second class of mutants, including the *rde-1* and *rde-4* loci, are defective for RNAi but show no other phenotypic abnormalities (Tabara et al., 1999). The *rde-1* and *rde-4* genes are required for the initiation of heritable RNAi, a phenomenon in which RNAi established by injection of dsRNA in a worm leads to heritable gene silencing in the F2 generation and beyond (Grishok et al., 2000). In contrast, *rde-2* and *mut-7* are not required for the initiation of heritable interference but are required downstream in the tissue where the interference occurs. Mello and colleagues have proposed that *rde-1* and *rde-4* respond to dsRNA by producing a secondary extragenic agent that is used by the downstream genes *rde-2* and *mut-7* to target specific mRNAs for posttranscriptional gene silencing (Grishok et al., 2000). In this view, *rde-1* and *rde-4* act as initiators of RNAi, whereas *rde-2* and *mut-7* are effectors. These authors propose that other stimuli that lead to gene silencing, such as the accumulation of transposons or repetitive DNA in the genome or the introduction of a transgene, are interpreted by a separate set of initiator genes that produce the same secondary extragenic agent.

In *Neurospora*, the *qde-3* gene, which is required for quelling (a form of posttranscriptional silencing in which an endogenous gene is silenced by the introduction of a transgenic copy of the gene), may be an example of an initiator gene that responds to the presence of a transgene (Cogoni and Macino, 1999b). *qde-3* is a member of the RecQ DNA helicase family, which includes

#To whom correspondence should be addressed (e-mail: phillip.zamore@umassmed.edu [P. D. Z.], ttuschl@mpibpc.gwdg.de [T. T.]).

the human genes for Bloom's syndrome and Werner's syndrome.

One candidate for the secondary extragenic agent itself is the 25 nucleotide-long RNAs associated with posttranscriptional gene silencing in plants (Hamilton and Baulcombe, 1999). These RNAs, which correspond to both the sense and antisense strands of the silenced gene, are only detected in plants undergoing silencing. The level of expression of these short RNAs also correlates with the extent of gene silencing. It remains to be shown if the 25 nt RNAs are the actual agents or merely the products of gene silencing.

Two other genes implicated in posttranscriptional gene silencing, *qde-1* in *Neurospora* (Cogoni and Macino, 1999a) and *ego-1* in *C. elegans* (Smardon et al., 2000), are homologous to a tomato protein that displays RNA-directed RNA-polymerase activity in vitro (Schiebel et al., 1993a, 1993b, 1998). RNA-directed RNA polymerases have been implicated in the initial formation of the silencing agent or in the amplification of dsRNA. Amplification of injected dsRNA by an endogenous RNA-directed RNA polymerase would help explain how a very small number of dsRNA molecules can inactivate a much larger population of mRNAs and how the dsRNA can apparently persist in the animal for many days and even into subsequent generations. *ego-1* mutants are defective for RNAi for maternally, but not zygotically, expressed mRNAs. Interestingly, *ego-1* is also required for germline development in *C. elegans* (Qiao et al., 1995).

Biochemical analysis of RNAi has become possible with the development of an in vitro *Drosophila* embryo lysate that recapitulates dsRNA-dependent silencing of gene expression (Tuschl et al., 1999). In the in vitro system, dsRNA—but not sense or asRNA—targets a corresponding mRNA for degradation yet does not affect the stability of an unrelated control mRNA. Furthermore, preincubation of the dsRNA in the lysate potentiates its activity for target mRNA degradation, suggesting that the dsRNA must be converted to an active form by binding proteins in the extract or by covalent modification (Tuschl et al., 1999).

Here, we use the in vitro system to analyze the requirements of RNAi and to determine the fate of the dsRNA and the mRNA. RNAi in vitro requires ATP but does not require either mRNA translation or recognition of the 7-methyl-guanosine cap of the targeted mRNA. The dsRNA but not single-stranded RNA is processed in vitro to a population of 21–23 nt species. Deamination of adenosines within the dsRNA does not appear to be required for formation of the 21–23 nt RNAs. Furthermore, we find that the mRNA is cleaved only in the region corresponding to the sequence of the dsRNA and that the mRNA is cleaved at 21–23 nt intervals, strongly suggesting that the 21–23 nt fragments from the dsRNA are targeting the cleavage of the mRNA.

Results and Discussion

RNAi Requires ATP

Drosophila embryo lysates faithfully recapitulate RNAi (Tuschl et al., 1999). Previously, dsRNA-mediated gene silencing was monitored by measuring the synthesis of

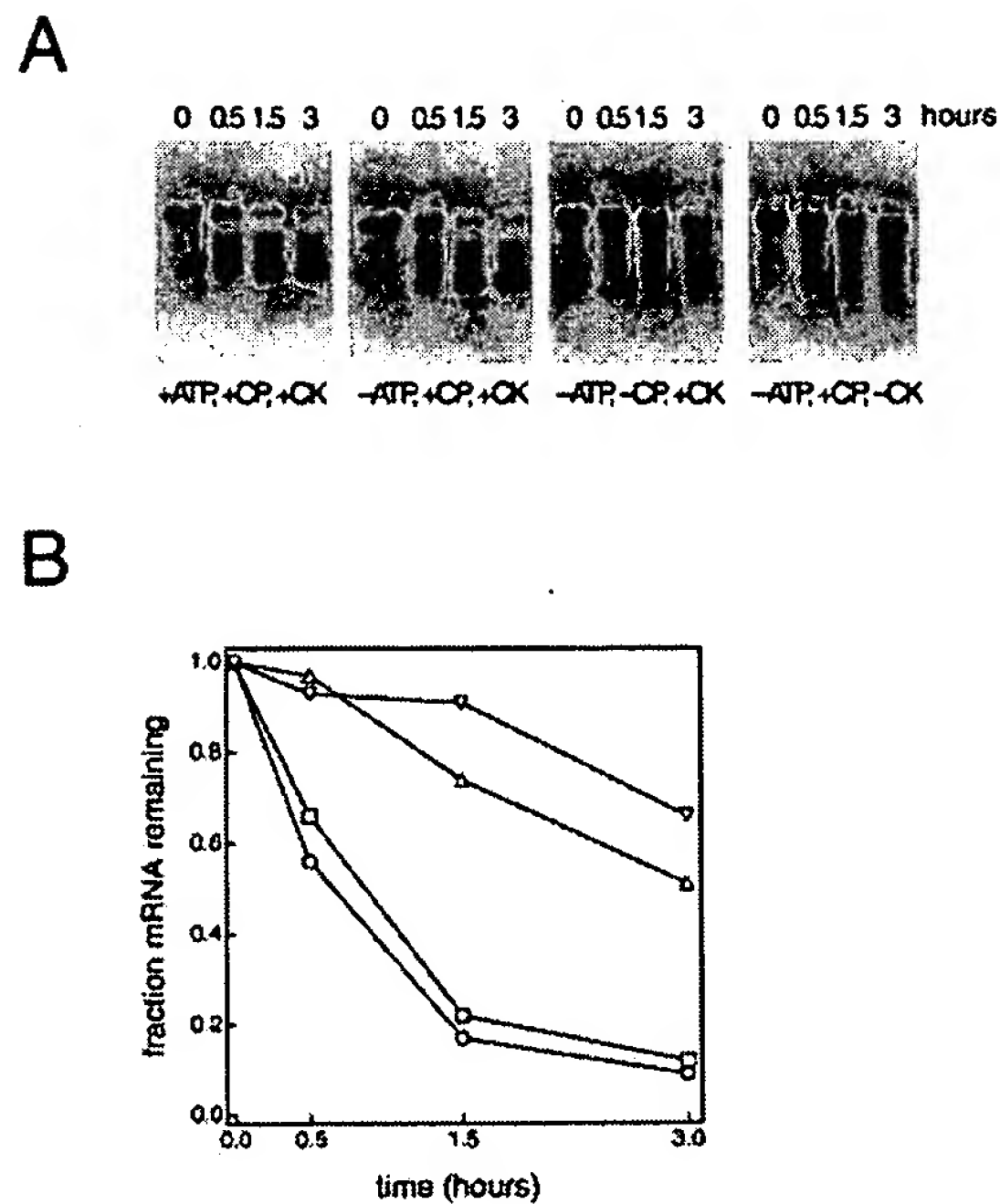


Figure 1. RNAi Requires ATP

(A) Denaturing agarose-gel analysis of 5'-³²P-radiolabeled *Rr-luc* mRNA incubated for the times indicated in an in vitro RNAi reaction with or without ATP, creatine phosphate (CP), or creatine kinase (CK), as indicated below each panel.

(B) Quantitation of the data in (A). Circles, +ATP, +CP, +CK; squares, -ATP, +CP, +CK; triangles, -ATP, -CP, +CK; inverted triangles, -ATP, +CP, -CK.

luciferase protein from the targeted mRNA. Thus, these RNAi reactions contained an ATP-regenerating system, needed for the efficient translation of the mRNA. To test if ATP was, in fact, required for RNAi, the lysates were depleted for ATP by treatment with hexokinase and glucose, which converts ATP to ADP, and RNAi was monitored directly by following the fate of ³²P-radiolabeled *Renilla reniformis* luciferase (*Rr-luc*) mRNA (Figure 1). Treatment with hexokinase and glucose reduced the endogenous ATP level in the lysate from 250 μM to below 10 μM (data not shown). ATP regeneration required both exogenous creatine phosphate and creatine kinase, which acts to transfer a high-energy phosphate from creatine phosphate to ADP. When ATP-depleted extracts were supplemented with either creatine phosphate or creatine kinase separately, no RNAi was observed. Therefore, RNAi requires ATP in vitro. When ATP, creatine phosphate, and creatine kinase were all added together to reactions containing the ATP-depleted lysate, dsRNA-dependent degradation of the *Rr-luc* mRNA was restored (Figure 1). The addition of exogenous ATP was not required for efficient RNAi in the depleted lysate, provided that both creatine phosphate and creatine kinase were present, demonstrating that the endogenous concentration (250 μM) of adenosine nucleotide is sufficient to support RNAi. RNAi with a *Photinus pyralis* luciferase (*Pp-luc*) mRNA was also ATP dependent (data not shown).

The stability of the *Rr-luc* mRNA in the absence of

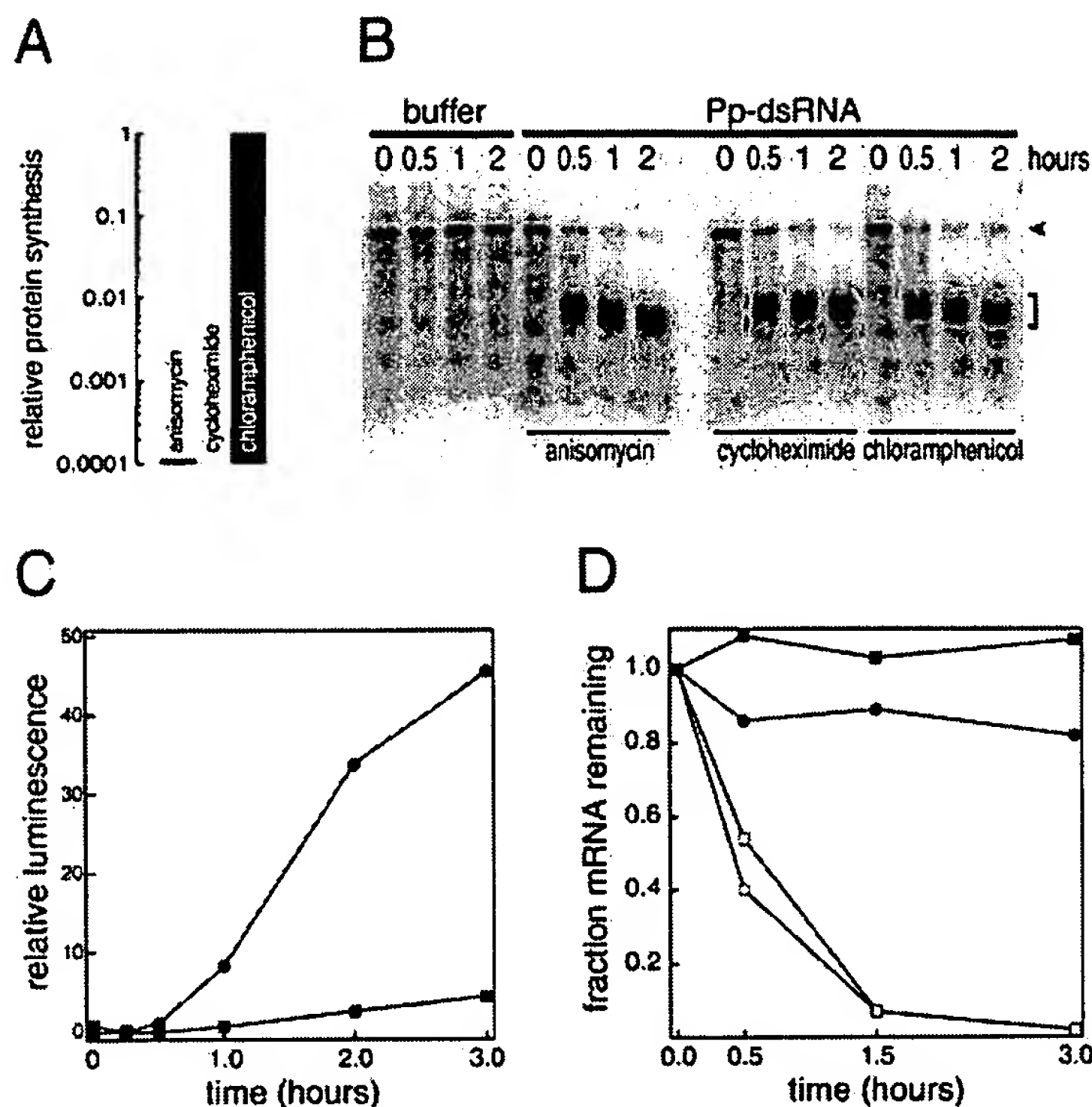


Figure 2. RNAi Does Not Require mRNA Translation

(A) Protein synthesis, as reflected by luciferase activity produced after incubation of *Rr-luc* mRNA in the in vitro RNAi reaction for 1 hr, in the presence of the protein synthesis inhibitors anisomycin, cycloheximide, or chloramphenicol, relative to a reaction without any inhibitor.

(B) Denaturing agarose-gel analysis of 5'-³²P-radiolabeled *Pp-luc* mRNA after incubation for the indicated times in a standard RNAi reaction with and without protein synthesis inhibitors. The arrowhead indicates the position of full-length mRNA in the gel, and the bracket marks the position of stable, 5' cleavage products.

(C) Translation of 7-methyl-guanosine- and adenosine-capped *Pp-luc* mRNAs (circles and squares, respectively) in the RNAi reaction in the absence of dsRNA, as measured by luciferase activity produced in a 1 hr incubation.

(D) Incubation in an RNAi reaction of uniformly ³²P-radiolabeled 7-methyl-guanosine-capped *Pp-luc* mRNA (circles) and adenosine-capped *Pp-luc* mRNA (squares), in the presence (open symbols) and absence (filled symbols) of 505 bp *Pp-luc* dsRNA.

Rr-dsRNA was reduced in ATP-depleted lysates relative to that observed when the energy regenerating system was included, but decay of the mRNA under these conditions did not display the rapid decay kinetics characteristic of RNAi in vitro, nor did it generate the stable mRNA cleavage products characteristic of dsRNA-directed RNAi (data not shown). These experiments do not establish if the ATP requirement for RNAi is direct, implicating ATP in one or more steps in the RNAi mechanism, or indirect, reflecting a role for ATP in maintaining high concentrations of another nucleoside triphosphate in the lysate.

Translation Is Not Required for RNAi In Vitro

The requirement for ATP suggested that RNAi might be coupled to mRNA translation, a highly energy-dependent process. To test this possibility, various inhibitors of protein synthesis were added to the reaction. We tested the eukaryotic translation inhibitors anisomycin, an inhibitor of initial peptide bond formation, cycloheximide, an inhibitor of peptide chain elongation, and puromycin, a tRNA mimic that causes premature termination of translation (Cundliffe, 1981). Each of these inhibitors reduced protein synthesis in the *Drosophila* lysate by more than 1,900-fold (Figure 2A; data not shown). In contrast, chloramphenicol, an inhibitor of *Drosophila* mitochondrial protein synthesis (Page and Orr-Weaver, 1997), had no effect on translation in the lysates (Figure 2A). Despite the presence of anisomycin, cycloheximide, or chloramphenicol, RNAi proceeded at normal efficiency (Figure 2B). Puromycin also did not perturb efficient RNAi (data not shown). Thus, protein synthesis is not required for RNAi in vitro.

Translational initiation is an ATP-dependent process that involves recognition of the 7-methyl guanosine cap of the mRNA (Merrick and Hershey, 1996; Kozak, 1999). The *Drosophila* lysate used to support RNAi in vitro also recapitulates the cap dependence of translation: *Pp-luc* mRNA with a 7-methyl-guanosine cap was translated greater than 10-fold more efficiently than was the same mRNA with an A(5')ppp(5')G cap (Figure 2C). Both RNAs were equally stable in the *Drosophila* lysate, showing that this difference in efficiency cannot be merely explained by more rapid decay of the mRNA with an adenosine cap (also see Gebauer et al., 1999). Although the translational machinery can discriminate between *Pp-luc* mRNAs with 7-methyl-guanosine and adenosine caps, the two mRNAs were equally susceptible to RNAi in the presence of *Pp*-dsRNA (Figure 2D). These results suggest that steps in cap recognition are not involved in RNAi.

dsRNA Is Processed to 21–23 Nucleotide Species

RNAs 25 nt in length are generated from both the sense and antisense strands of genes undergoing posttranscriptional gene silencing in plants (Hamilton and Baulcombe, 1999). We find that dsRNA is also processed to small RNA fragments (Figures 3A and 3B). When incubated in lysate, approximately 15% of the input radioactivity of both the 501 bp *Rr*-dsRNA and the 505 bp *Pp*-dsRNA appeared in 21 to 23 nt RNA fragments. Because the dsRNAs are more than 500 bp in length, the 15% yield of fragments implies that multiple 21–23 nt RNAs are produced from each full-length dsRNA molecule. No other stable products were detected. The small RNA species were produced from dsRNAs in which both

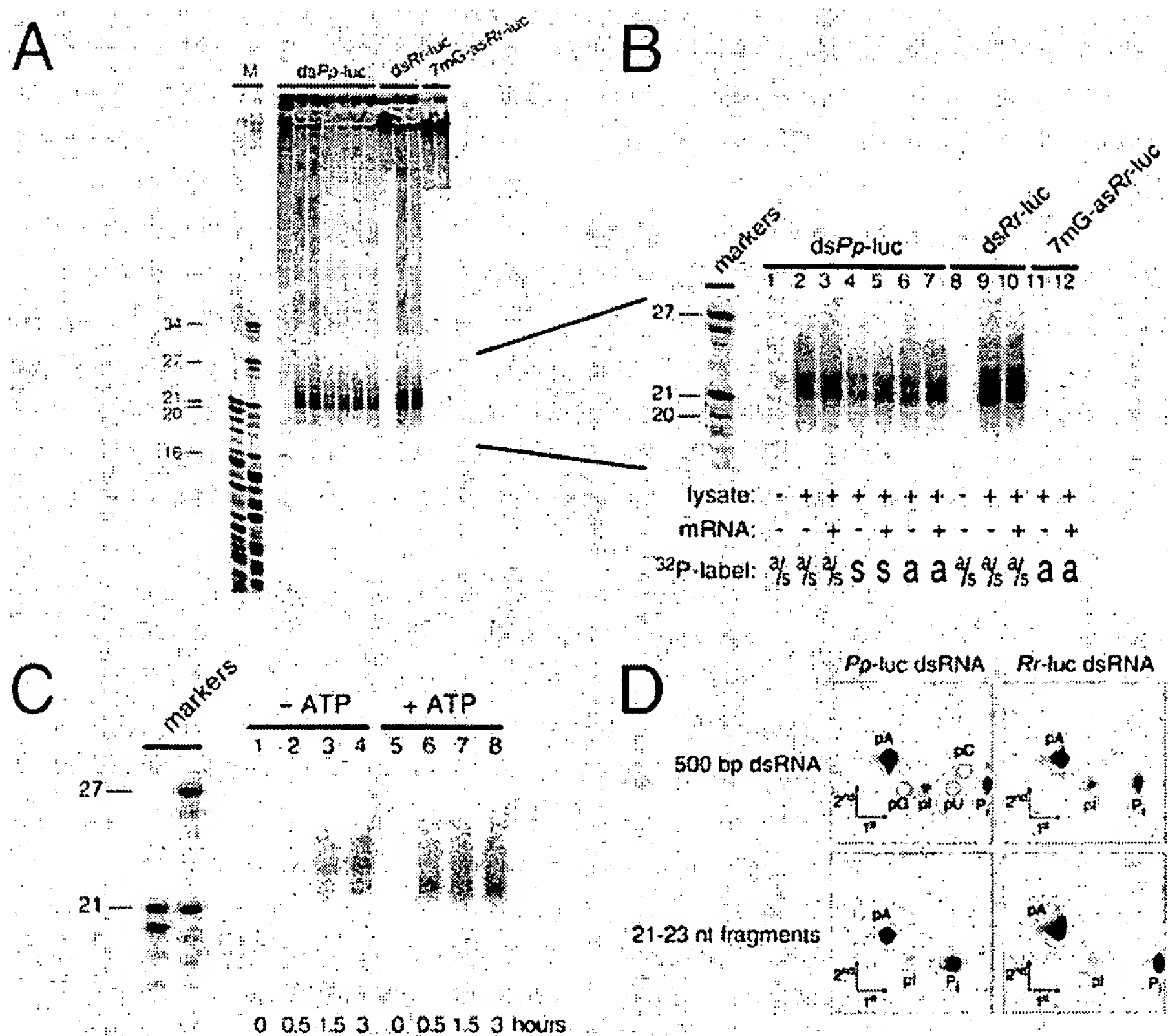


Figure 3. 21–23 nt RNA Fragments Are Produced upon Incubation of dsRNA in *Drosophila* Embryo Lysate

(A) Denaturing acrylamide-gel analysis of the products formed in a 2 hr incubation of uniformly ³²P-radiolabeled dsRNAs or capped asRNA in lysate under standard RNAi conditions, in the presence or absence of target mRNAs.

(B) An enlargement of the gel in (A) corresponding to 17 to 27 nt. For *Pp*-dsRNA, the sense (lanes 4 and 5) or the antisense (lanes 6 and 7) or both strands (lanes 1, 2, and 3) were labeled. For *Rr*-luc dsRNA, both strands were radioactive (lanes 8, 9, and 10).

(C) An enlargement of the 17 to 27 nt region of a gel showing the products formed upon incubation of uniformly ³²P-radiolabeled dsRNAs in lysate without and with ATP.

(D) Adenosine deamination in full-length dsRNA and the 21–23 nt RNA species assessed by two-dimensional thin-layer chromatography. Circles correspond to positions of unlabeled 5'-nucleotide monophosphate standards visualized under UV light. Inorganic phosphate (P_i) was produced by the degradation of mononucleotides by phosphatases that contaminate commercially available nuclease P1 (Auxilien et al., 1996).

strands were uniformly ³²P-radiolabeled (Figure 3B, lanes 2, 3, 9, and 10). Formation of the 21–23 nt RNAs from the dsRNA did not require the presence of the corresponding mRNA (Figure 3B, compare lane 2 with lane 3 and lane 9 with lane 10), demonstrating that the small RNA species is generated by processing of the dsRNA, rather than as a product of dsRNA-targeted mRNA degradation. We note that 22 nucleotides corresponds to two turns of an A-form RNA–RNA helix.

When dsRNAs radiolabeled within either the sense or the antisense strand were incubated with lysate in a standard RNAi reaction, 21–23 nt RNAs were generated with comparable efficiency (Figure 3B, compare lanes 4 and 6). These data support the idea that the 21–23 nt RNAs are generated by symmetric processing of the dsRNA. A variety of data support the idea that the 21–23 nt RNA is efficiently generated only from dsRNA and is not the consequence of an interaction between single-stranded RNA and the dsRNA. First, a ³²P-radiolabeled 505 nt *Pp*-luc sense RNA or asRNA was not efficiently

converted to the 21–23 nt product when it was incubated with 5 nM nonradioactive 505 bp *Pp*-dsRNA (data not shown). Second, in the absence of mRNA, a 501 nt 7-methyl-guanosine-capped *Rr*-asRNA produced only a barely detectable amount of 21–23 nt RNA (Figure 3B, lane 11; capped single-stranded RNAs are as stable in the lysate as dsRNA [Tuschl et al., 1999]), probably due to a small amount of dsRNA contaminating the antisense preparation. However, when *Rr*-luc mRNA was included in the reaction with the ³²P-radiolabeled, capped *Rr*-asRNA, a small amount of 21–23 nt product was generated, corresponding to 4% of the amount of 21–23 nt RNA produced from an equimolar amount of *Rr*-dsRNA. This result is unlikely to reflect the presence of contaminating dsRNA in the *Rr*-asRNA preparation, since significantly more product was generated from the asRNA in the presence of the *Rr*-luc mRNA than in the absence (compare lanes 12 and 11). Instead, the data suggest that asRNA can interact with the complementary mRNA sequences to form dsRNA in the reaction and that the

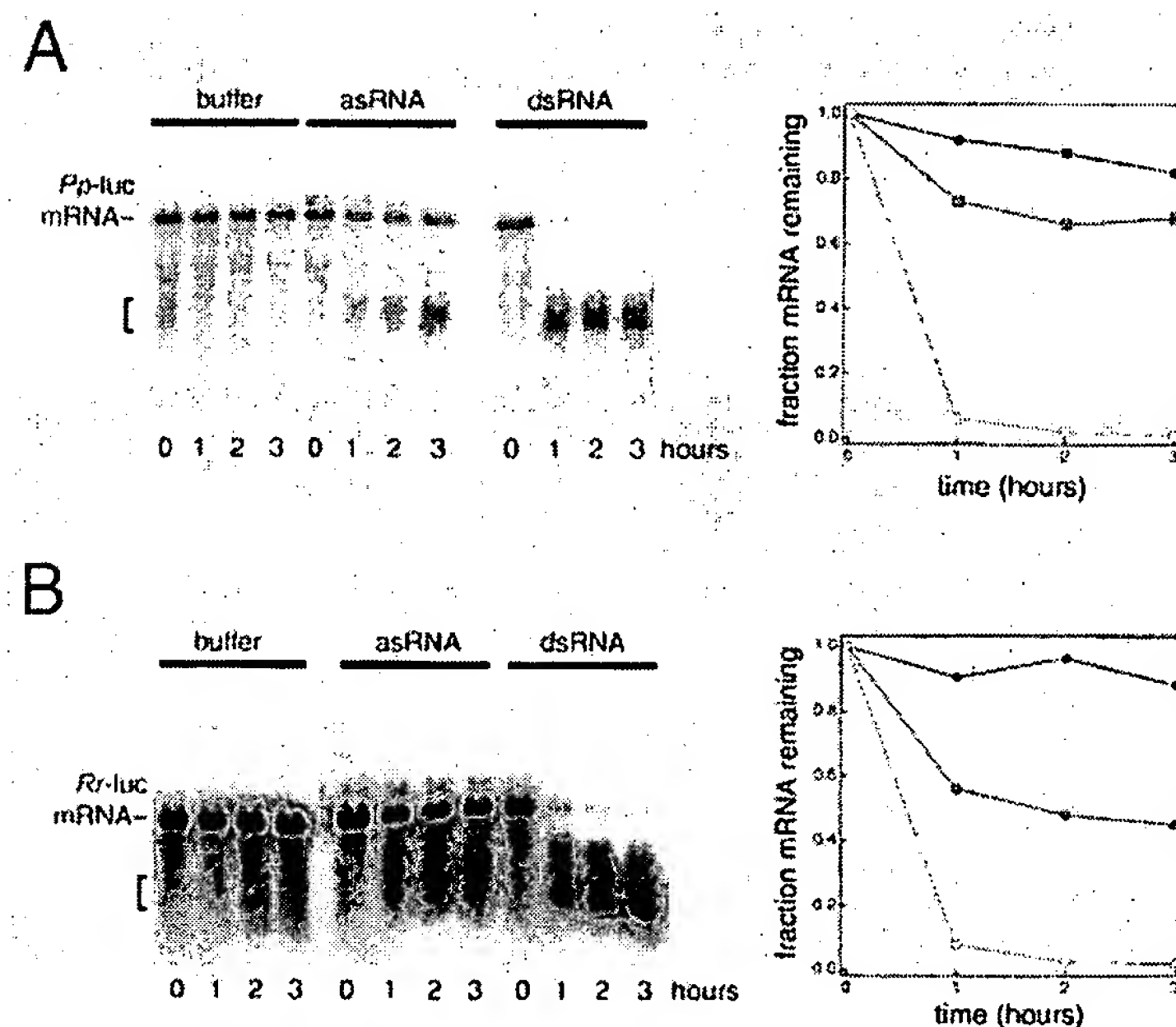


Figure 4. asRNA Causes a Small Amount of RNAi In Vitro

(A) Denaturing agarose-gel analysis of *Pp-luc* mRNA incubated in a standard RNAi reaction with buffer, 505 nt *Pp-asRNA*, or 505 bp *Pp-dsRNA* for the times indicated.

(B) The same analysis for the *Rr-luc* mRNA. Quantitation of the gel data in both (A) and (B) is given to the right of each panel. Buffer, black symbols; asRNA, blue symbols; dsRNA, red symbols.

resulting dsRNA is subsequently processed to the small RNA species. *Rr-asRNA* can support a low level of bona fide RNAi in vitro (see below), consistent with this explanation.

We next asked if production of the 21–23 nt RNAs from dsRNA required ATP (Figure 3C). When the 505 bp *Pp-dsRNA* was incubated in a lysate depleted for ATP by treatment with hexokinase and glucose, 21–23 nt RNA was produced (lanes 1–4, “–ATP”), albeit six times slower than when ATP was regenerated in the depleted lysate by the inclusion of creatine kinase and creatine phosphate (lanes 5–8, “+ ATP”). Therefore, ATP may not be required for production of the 21–23 nt RNA species but may instead simply enhance its formation. Alternatively, ATP may be required for processing of the dsRNA, but at a concentration less than that remaining after hexokinase treatment. We do not yet understand the molecular basis for the slower mobility of the small RNA fragments generated in the ATP-depleted lysate.

Wagner and Sun (1998) and Sharp (1999) have speculated that the requirement for dsRNA in gene silencing by RNAi reflects the involvement of a dsRNA-specific adenosine deaminase in the process. dsRNA adenosine deaminases unwind dsRNA by converting adenosine to inosine, which does not base pair with uracil. dsRNA adenosine deaminases function in the posttranscriptional editing of mRNA (reviewed by Bass, 1997). To test for the involvement of dsRNA adenosine deaminase in RNAi, we examined the degree of conversion of adenosine to inosine in the 501 bp *Rr-luc* and 505 bp *Pp-luc* dsRNAs after incubation with *Drosophila* embryo lysate in a standard in vitro RNAi reaction (Figure 3D). We also determined the degree of adenosine deamination in the 21–23 nt species. The full-length dsRNA radiolabeled with [³²P]-adenosine was incubated in the lysate, and both the full-length dsRNA and the 21–23 nt RNA products were purified from a denaturing acrylamide gel,

cleaved to mononucleotides with nuclease P1, and analyzed by two-dimensional thin-layer chromatography.

A significant fraction of the adenosines in the full-length dsRNA were converted to inosine after 2 hr (3.1% and 5.6% conversion for *Pp-luc* and *Rr-luc* dsRNAs, respectively). In contrast, only 0.4% (*Pp-dsRNA*) or 0.7% (*Rr-dsRNA*) of the adenosines in the 21–23 nt species were deaminated. These data imply that fewer than 1 in 27 molecules of the 21–23 nt RNA species contain an inosine. Therefore, it is unlikely that dsRNA-dependent adenosine deamination within the 21–23 nt species is required for its production.

asRNA Generates a Small Amount of RNAi In Vitro

When mRNA was ³²P-radiolabeled within the 5′-7-methyl-guanosine cap, stable 5′ decay products accumulated during the RNAi reaction (see, for example, Figures 1A and 2B). Such stable 5′ decay products were observed for both the *Pp-luc* and *Rr-luc* mRNAs when they were incubated with their cognate dsRNAs (indicated by the brackets in Figures 4A and 4B). Previously, we reported that efficient RNAi does not occur when asRNA is used in place of dsRNA (Tuschl et al., 1999). Nevertheless, mRNA was measurably less stable when incubated with asRNA than with buffer (Figures 4A and 4B). This was particularly evident for the *Rr-luc* mRNA: approximately 90% of the RNA remained intact after a 3 hr incubation in lysate, but only 50% when asRNA was added. Less than 5% remained when dsRNA was added. Interestingly, the decrease in mRNA stability caused by asRNA was accompanied by the formation of a small amount of the stable 5′ decay products characteristic of the RNAi reaction with dsRNA. This finding parallels the observation that a small amount of 21–23 nt product formed from the asRNA when it was incubated with the mRNA (see above) and lends strength to the idea that asRNA can enter the RNAi pathway, albeit inefficiently.

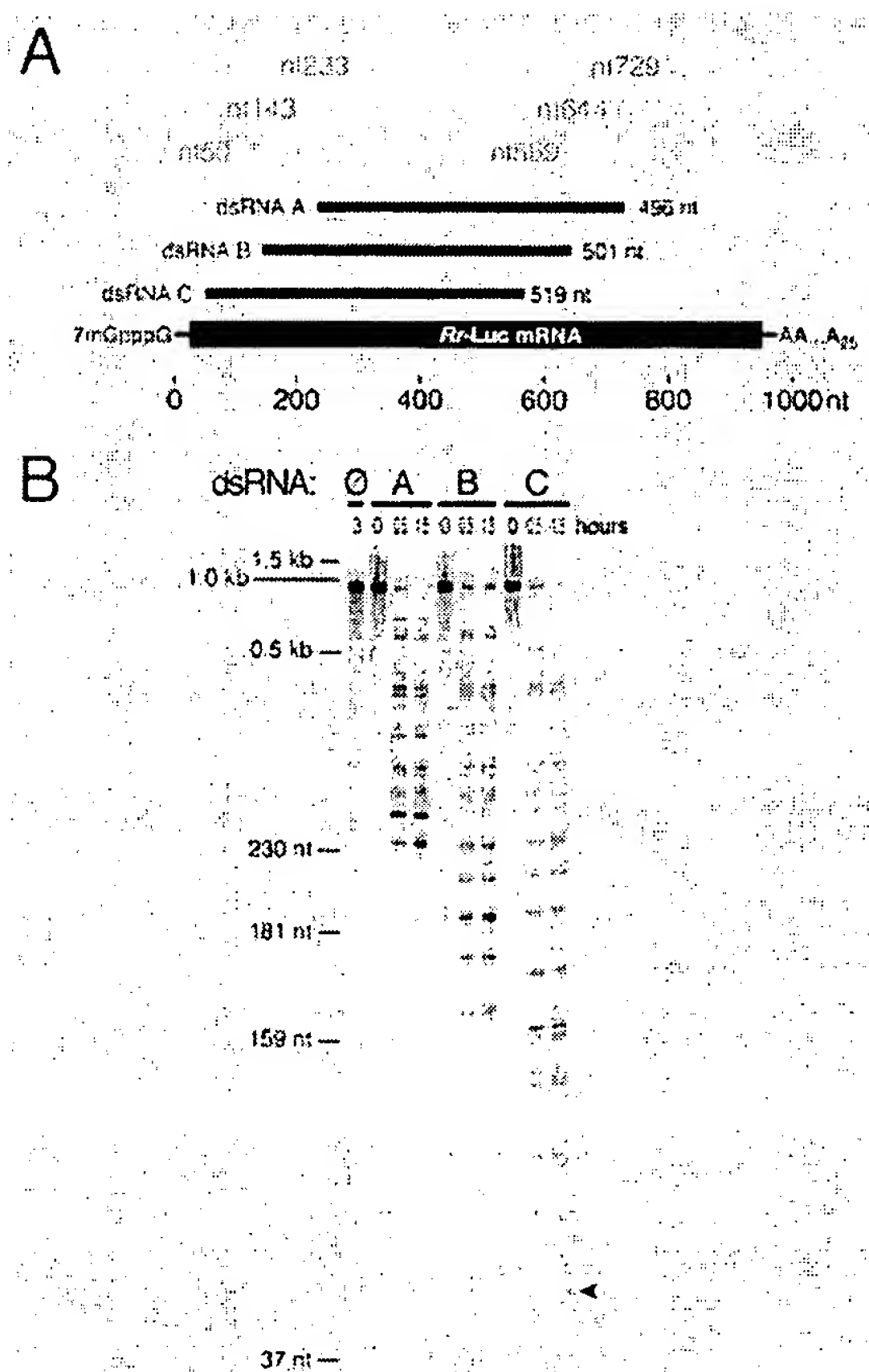


Figure 5. The dsRNA Determines the Boundaries of the Cleavage Products

(A) Schematic of the positions of the three dsRNAs, A, B, and C, relative to the *Rr-luc* mRNA.

(B) Denaturing acrylamide-gel analysis of the stable, 5' cleavage products produced after incubation of the *Rr-luc* mRNA for the indicated times with each of the three dsRNAs, A, B, and C, or with buffer (zero with strikethrough). The positions of RNA markers radiolabeled within their 5' cap is shown at left. The arrowhead denotes a faint cleavage site that is indicated with an open blue circle in Figure 6B.

mRNA Cleavage Sites Are Determined by the Sequence of the dsRNA

The sites of mRNA cleavage were examined using three different dsRNAs, "A," "B," and "C," displaced along the *Rr-luc* sequence by approximately 100 nt. The positions of these relative to the *Rr-luc* mRNA sequence are shown (Figure 5A). Each of the three dsRNAs was incubated in a standard RNAi reaction with *Rr-luc* mRNA ³²P-radiolabeled within the 5' cap (Figure 5B). In the absence of dsRNA, no stable 5' cleavage products were detected for the mRNA, even after 3 hr of incubation in lysate. In contrast, after a 20 min incubation, each of the three dsRNAs produced a ladder of bands corresponding to a set of mRNA cleavage products characteristic for that particular dsRNA. For each dsRNA, the stable, 5' mRNA cleavage products were restricted to the region of the *Rr-luc* mRNA that corresponded to the dsRNA (Figures 5B and 6). For dsRNA A, the lengths of the 5' cleavage products ranged from 236 to just under

~750 nt; dsRNA A spans nucleotides 233 to 729 of the *Rr-luc* mRNA. Incubation of the mRNA with dsRNA B produced mRNA 5' cleavage products ranging in length from 150 to ~600 nt; dsRNA B spans nucleotides 143 to 644 of the mRNA. Finally, dsRNA C produced mRNA cleavage products from 66 to ~500 nt in length. This dsRNA spans nucleotides 50 to 569 of the *Rr-luc* mRNA. Therefore, the dsRNA not only provides specificity for the RNAi reaction, selecting which mRNA from the total cellular mRNA pool will be degraded, but also determines the boundaries of cleavage along the mRNA sequence.

The mRNA Is Cleaved at 21–23 Nucleotide Intervals

To gain further insight into the mechanism of RNAi, we mapped the positions of several mRNA cleavage sites for each of the three dsRNAs (Figure 6). Remarkably, most of the cleavages occurred at 21–23 nt intervals (Figure 6A). This spacing is especially striking in light of our observation that the dsRNA is processed to a 21–23 nt RNA species and the finding of Hamilton and Baulcombe that a 25 nt RNA correlates with posttranscriptional gene silencing in plants (Hamilton and Baulcombe, 1999). Of the 16 cleavage sites we mapped (two for dsRNA A, five for dsRNA B, and nine for dsRNA C), all but two reflect the 21–23 nt interval. One of the two exceptional cleavages was a weak cleavage site produced by dsRNA C (indicated by an arrowhead in Figure 5B and an open blue circle in Figure 6B). This cleavage occurred 32 nt 5' to the next cleavage site. The other exception is particularly intriguing. After four cleavages spaced 21–23 nt apart, dsRNA C caused cleavage of the mRNA just 9 nt 3' to the previous cleavage site (Figures 6A and 6B, red arrowhead). This cleavage occurred in a run of seven uracil residues and appears to "reset" the ruler for cleavage; the next cleavage site was 21–23 nt 3' to the exceptional site. The three subsequent cleavage sites that we mapped were also spaced 21–23 nt apart. Curiously, of the sixteen cleavage sites mapped for the three different dsRNAs, fourteen occur at uracil residues. We do not yet understand the significance of this finding, but it suggests that mRNA cleavage is determined by a process that measures 21–23 nt intervals and that has a sequence preference for cleavage at uracil. In preliminary experiments, the 21–23 nt RNA species produced by incubation of ~500 bp dsRNA in the lysate caused sequence-specific interference *in vitro* when isolated from an acrylamide gel and added to a new RNAi reaction in place of the full-length dsRNA (our unpublished data).

A Model for dsRNA-Directed mRNA Cleavage

Our biochemical data, together with recent genetic experiments in *C. elegans* and *Neurospora* (Cogoni and Macino, 1999a; Ketting et al., 1999; Tabara et al., 1999; Grishok et al., 2000), suggest a model for how dsRNA targets mRNA for destruction (Figure 7). In this model, the dsRNA is first cleaved to 21 to 23 nt long fragments in a process likely to involve genes such as the *C. elegans* loci *rde-1* and *rde-4*. The resulting fragments, probably as short asRNAs bound by RNAi-specific proteins, would then pair with the mRNA and recruit a nuclease that cleaves the mRNA. Alternatively, strand exchange could occur in a protein-RNA complex that transiently

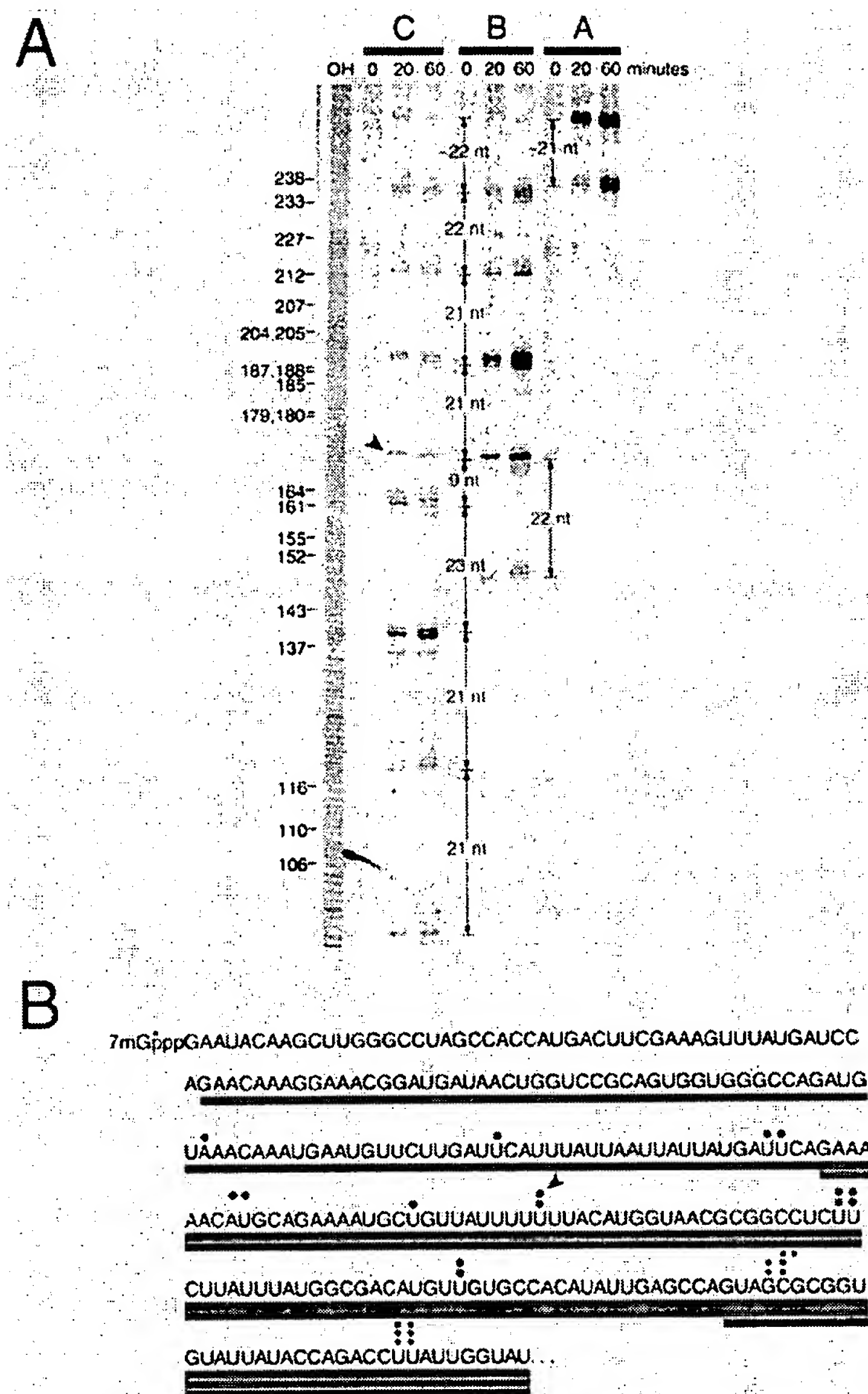


Figure 6. The mRNA is cleaved in 21–23 nt intervals

(A) High-resolution denaturing acrylamide-gel analysis of a subset of the 5' cleavage products described in Figure 5B. The positions of some of the partial T1 digestion products of *Rr-luc* mRNA are indicated at left. "OH" marks the lane in which a partial base-hydrolysis ladder was loaded.

(B) The cleavage sites in (A) mapped onto the first 267 nt of the *Rr-luc* mRNA. The blue bar below the sequence indicates the position of dsRNA C, and blue circles indicate the position of cleavage sites caused by this dsRNA. The green bar denotes the position of dsRNA B, and green circles, the cleavage sites. The magenta bar indicates the position of dsRNA A, and magenta circles, the cleavages. An exceptional cleavage within a run of seven uracils is marked with a red arrowhead in both (A) and (B).

holds a 21–23 nt dsRNA fragment close to the mRNA. Separation of the two strands of the dsRNA following fragmentation might be assisted by an ATP-dependent RNA helicase, explaining the ATP enhancement of 21–23 nt RNA production we observed.

We envision that each small RNA fragment produces one, or at most two, cleavages in the mRNA, perhaps at the 5' or 3' ends of the 21–23 nt fragment. The small RNAs may be amplified by an RNA-directed RNA polymerase such as that encoded by the *ego-1* gene in *C. elegans* (Smardon et al., 2000) or the *qde-1* gene in *Neurospora* (Cogoni and Macino, 1999a), producing long-lasting posttranscriptional gene silencing in the absence of the dsRNA that initiated the RNAi effect. Heritable RNAi in *C. elegans* requires the *rde-1* and *rde-4* genes to initiate but not to persist in subsequent generations. The *rde-2*, *rde-3*, and *mut-7* genes in *C. elegans* are required in the tissue where RNAi occurs but are

not required for initiation of heritable RNAi (Grishok et al., 2000). These "effector" genes (Grishok et al., 2000) are likely to encode proteins functioning in the actual selection of mRNA targets and in their subsequent cleavage. ATP may be required at any of a number of steps during RNAi, including complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21–23 nt RNAs with the target mRNA, mRNA cleavage, and recycling of the targeting complex. Testing these ideas with the in vitro RNAi system will be an important challenge for the future.

Experimental Procedures

In Vitro RNAi

In vitro RNAi reactions and lysate preparation were as described previously (Tuschl et al., 1999) except that the reaction contained 0.03 μ g/ml creatine kinase, 25 mM creatine phosphate (Fluka), and 1 mM ATP. Creatine phosphate was freshly dissolved at 500 mM in

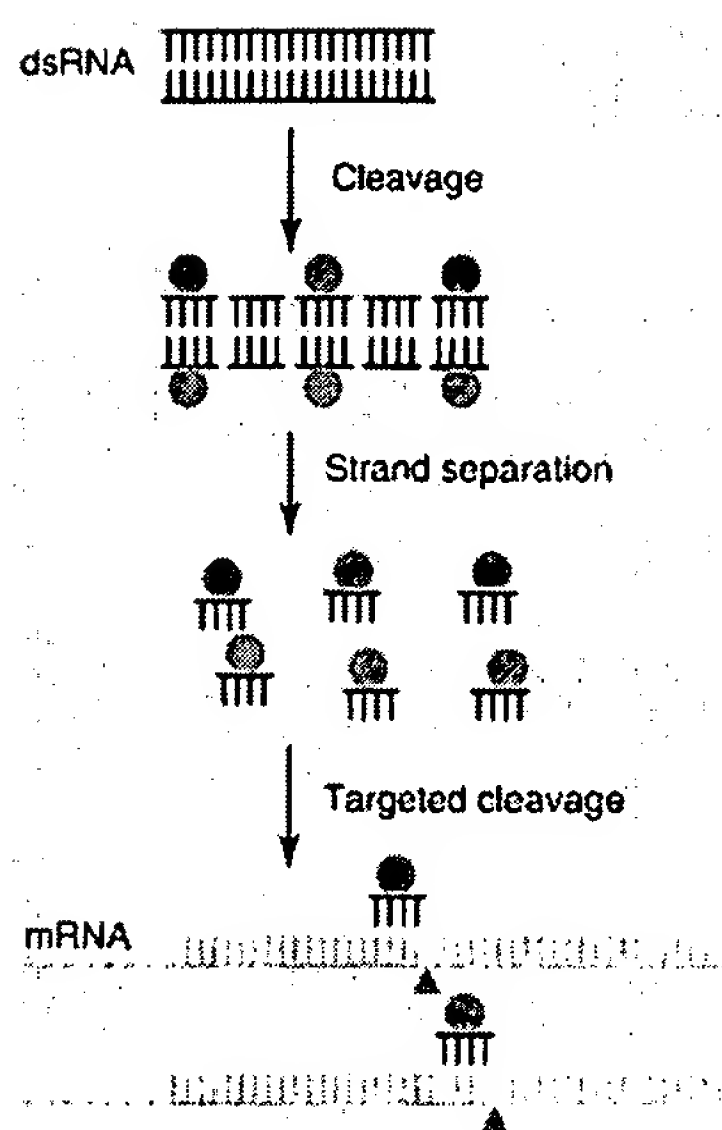


Figure 7. Proposed Model for RNAi

RNAi is envisioned to begin with cleavage of the dsRNA to 21–23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP-dependent helicase, possibly a component of the initial complex, to 21–23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (ochre circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

water for each experiment. GTP was omitted from the reactions, except in Figures 2 and 3.

RNA Synthesis

Pp-luc and *Rr-luc* mRNAs and *Pp-* and *Rr-dsRNAs* (including dsRNA B in Figure 6) were synthesized by in vitro transcription as described previously (Tuschl et al., 1999). To generate transcription templates for dsRNA C, the 5' sense RNA primer was gcgtaatcagactcactata GAACAAAGGAAACGGATGAT and the 3' sense RNA primer was GAAGAAGTTATTCTCCAAA; the 5' asRNA primer was gcgtaatcagactcactataGAAGAAGTTATTCTCCAAA and the 3' asRNA primer was GAACAAAGGAAACGGATGAT. For dsRNA A, the 5' sense RNA primer was gcgtaatcagactcactataGTAGCGCGGTGTATTATACC and the 3' sense RNA primer was GTACAACGTCAGGTTACCA; the 5' asRNA primer was gcgtaatcagactcactataGTACAACGTCAGGTTTACCA and the 3' asRNA primer was GTAGCGCGGTGTATTATACC (lowercase, T7 promoter sequence).

mRNAs were 5' end labeled using guanylyl transferase (Gibco/BRL), S-adenosyl methionine (Sigma), and α - 32 P-GTP (3000 Ci/mmol; New England Nuclear) according to the manufacturer's directions. Radiolabeled RNAs were purified by poly(A) selection using the Poly(A) Tract III kit (Promega). Nonradioactive 7-methyl-guanosine- and adenosine-capped RNAs were synthesized in in vitro transcription reactions with a 5-fold excess of 7-methyl-G(5')ppp(5')G or A(5')ppp(5')G relative to GTP. Cap analogs were purchased from New England Biolabs.

ATP Depletion and Protein Synthesis Inhibition

ATP was depleted by incubating the lysate for 10 min at 25°C with 2 mM glucose and 0.1 U/ μ l hexokinase (Sigma). Protein synthesis inhibitors were purchased from Sigma and dissolved in absolute ethanol as 250-fold concentrated stocks. The final concentrations of inhibitors in the reaction were anisomycin, 53 μ g/ml; cycloheximide, 100 μ g/ml; and chloramphenicol, 100 mg/ml. Relative protein

synthesis was determined by measuring the activity of *Rr luciferase* protein produced by translation of the *Rr-luc* mRNA in the RNAi reaction after 1 hr as described previously (Tuschl et al., 1999).

Analysis of dsRNA Processing

Internally α - 32 P-ATP-labeled dsRNAs (505 bp *Pp-luc* or 501 *Rr-luc*) or 7-methyl-guanosine-capped *Rr-luc* antisense RNA (501 nt) were incubated at 5 nM final concentration in the presence or absence of unlabeled mRNAs in *Drosophila* lysate for 2 hr in standard conditions. Reactions were stopped by the addition of 2 \times proteinase K buffer and deproteinized as described previously (Tuschl et al., 1999). Products were analyzed by electrophoresis in 15% or 18% polyacrylamide sequencing gels. Length standards were generated by complete RNase T1 digestion of α - 32 P-ATP-labeled 501 nt *Rr-luc* sense RNA and asRNA.

For analysis of mRNA cleavage, 5'- 32 P-radiolabeled mRNA (described above) was incubated with dsRNA as described previously (Tuschl et al., 1999) and analyzed by electrophoresis in 5% (Figure 5B) and 6% (Figure 6C) polyacrylamide sequencing gels. Length standards included commercially available RNA size standards (FMC Bioproducts) radiolabeled with guanylyl transferase as described above and partial base hydrolysis and RNase T1 ladders generated from the 5'-radiolabeled mRNA.

Deamination Assay

Internally α - 32 P-ATP-labeled dsRNAs (5 nM) were incubated in *Drosophila* lysate for 2 hr at standard conditions. After deproteinization, samples were run on 12% sequencing gels to separate full-length dsRNAs from the 21–23 nt products. RNAs were eluted from the gel slices in 0.3 M NaCl overnight, ethanol precipitated, collected by centrifugation, and redissolved in 20 μ l water. The RNA was hydrolyzed into nucleoside 5' phosphates with nuclease P1 (10 μ l reaction containing 8 μ l RNA in water, 30 mM KOAc [pH 5.3], 10 mM ZnSO₄, and 10 μ g or 3 units nuclease P1, for 3 hr at 50°C). Samples (1 μ l) were cospotted with nonradioactive 5' mononucleotides (0.05 O. D. units [A_{260}] of pA, pC, pG, pI, and pU) on cellulose HPTLC plates (EM Merck) and separated in the first dimension in isobutyric acid/25% ammonia/water (66/1/33, v/v/v) and in the second dimension in 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol (100/60/2, v/w/v; Silberklang et al., 1979). Migration of the nonradioactive internal standards was determined by UV shadowing.

Acknowledgments

We acknowledge Heike Taubner, Sayda M. Elbashir, and Winfried Lendeckel for assistance, and Herbert Jäckle and his research group and members of the Bartel laboratory for their support and advice. The authors thank Terri Orr-Weaver for providing fly resources and Marlene Castle for maintaining population cages. This work was supported by a German BMBF Biofuture grant number 0311856 (T. T.), by the National Institutes of Health through a United States Public Health Service MERIT award, the National Science Foundation, and partially by the National Cancer Institute through a Cancer Center Support core grant (P. A. S.), and by the Searle Scholars Program/The Chicago Community Trust (D. P. B.).

Received March 2, 2000; revised March 10, 2000.

References

- Auxilien, S., Crain, P.F., Trewyn, R.W., and Grosjean, H. (1996). Mechanism, specificity and general properties of the yeast enzyme catalysing the formation of inosine 34 in the anticodon of transfer RNA. *J. Mol. Biol.* 262, 437–458.
- Bass, B.L. (1997). RNA editing and hypermutation by adenosine deamination [published erratum appears in *Trends Biochem. Sci.* 1997 Jul;22(7):278]. *Trends Biochem. Sci.* 22, 157–162.
- Baulcombe, D.C. (1999). Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant Biol.* 2, 109–113.
- Cogoni, C., and Macino, G. (1997). Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced

- gene silencing in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* **94**, 10233–10238.
- Cogoni, C., and Macino, G. (1999a). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* **399**, 166–169.
- Cogoni, C., and Macino, G. (1999b). Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. *Science* **286**, 2342–2344.
- Cogoni, C., Irelan, J.T., Schumacher, M., Schmidhauser, T.J., Selker, E.U., and Macino, G. (1996). Transgene silencing of the *ai-1* gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. *EMBO J.* **15**, 3153–3163.
- Cundliffe, E. (1981). Antibiotic inhibitors of ribosome function. In *The Molecular Basis of Antibiotic Action*, E. Gale et al., eds. (New York: Wiley), pp. 402–547.
- Fire, A. (1999). RNA-triggered gene silencing. *Trends Genet.* **15**, 358–363.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.
- Gebauer, F., Corona, D.F., Preiss, T., Becker, P.B., and Hentze, M.W. (1999). Translational control of dosage compensation in *Drosophila* by Sex-lethal: cooperative silencing via the 5' and 3' UTRs of *msl-2* mRNA is independent of the poly(A) tail. *EMBO J.* **18**, 6146–6154.
- Grant, S.R. (1999). Dissecting the mechanisms of posttranscriptional gene silencing: divide and conquer. *Cell* **96**, 303–306.
- Grishok, A., Tabara, H., and Mello, C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science*, in press.
- Guo, S., and Kemphues, K.J. (1995). *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611–620.
- Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952.
- Hunter, C.P. (1999). A touch of elegance with RNAi. *Curr. Biol.* **9**, R440–R442.
- Hunter, C. (2000). Gene silencing: shrinking the black box of RNAi. *Curr. Biol.* **10**, R137–R140.
- Kennerdell, J.R., and Carthew, R.W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* **95**, 1017–1026.
- Ketting, R.F., Haverkamp, T.H., van Luenen, H.G., and Plasterk, R.H. (1999). Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**, 133–141.
- Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. *Gene* **234**, 187–208.
- Lohmann, J.U., Endl, I., and Bosch, T.C. (1999). Silencing of developmental genes in *Hydra*. *Dev. Biol.* **214**, 211–214.
- Merrick, W., and Hershey, J. (1996). The pathway and mechanism of eukaryotic protein synthesis. In *Translational Control*, J. Hershey et al., eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 31–69.
- Misquitta, L., and Paterson, B.M. (1999). Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc. Natl. Acad. Sci. USA* **96**, 1451–1456.
- Montgomery, M.K., and Fire, A. (1998). Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. *Trends Genet.* **14**, 255–258.
- Montgomery, M.K., Xu, S., and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **95**, 15502–15507.
- Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA* **95**, 14687–14692.
- Page, A.W., and Orr-Weaver, T.L. (1997). Activation of the meiotic divisions in *Drosophila* oocytes. *Dev. Biol.* **183**, 195–207.
- Qiao, L., Lissemore, J.L., Shu, P., Smardon, A., Gelber, M.B., and Maine, E.M. (1995). Enhancers of *glp-1*, a gene required for cell-signaling in *Caenorhabditis elegans*, define a set of genes required for germline development. *Genetics* **141**, 551–569.
- Ratcliff, F.G., MacFarlane, S.A., and Baulcombe, D.C. (1999). Gene silencing without DNA. RNA-mediated cross-protection between viruses. *Plant Cell* **11**, 1207–1216.
- Sánchez-Alvarado, A., and Newmark, P.A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* **96**, 5049–5054.
- Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sanger, H.L. (1993a). RNA-directed RNA polymerase from tomato leaves. I. Purification and physical properties. *J. Biol. Chem.* **268**, 11851–11857.
- Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sanger, H.L. (1993b). RNA-directed RNA polymerase from tomato leaves. II. Catalytic in vitro properties. *J. Biol. Chem.* **268**, 11858–11867.
- Schiebel, W., Pelissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sanger, H.L., and Wassenegger, M. (1998). Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. *Plant Cell* **10**, 2087–2101.
- Sharp, P.A. (1999). RNAi and double-strand RNA. *Genes Dev.* **13**, 139–141.
- Silberklang, M., Gillum, A.M., and RajBhandary, U.L. (1979). Use of in vitro ³²P labeling in the sequence analysis of nonradioactive tRNAs. *Methods Enzymol.* **59**, 58–109.
- Smardon, A., Spoerke, J., Stacey, S., Klein, M., Mackin, N., and Maine, E. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* **10**, 169–178.
- Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99**, 123–132.
- Timmons, L., and Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.
- Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., and Sharp, P.A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev.* **13**, 3191–3197.
- Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C., and Vernhettes, S. (1998). Transgene-induced gene silencing in plants. *Plant J.* **16**, 651–659.
- Wagner, R., and Sun, L. (1998). Functional genomics: double-stranded RNA poses puzzle. *Nature* **391**, 744–745.
- Wargelius, A., Ellingsen, S., and Fjose, A. (1999). Double-stranded RNA induces specific developmental defects in zebrafish embryos. *Biochem. Biophys. Res. Commun.* **263**, 156–161.
- Waterhouse, P.M., Graham, M.W., and Wang, M.B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl. Acad. Sci. USA* **95**, 13959–13964.
- Waterhouse, P.M., Smith, N.A., and Wang, M.-B. (1999). Virus resistance and gene silencing: killing the messenger. *Trends Plant Sci.* **4**, 452–457.
- Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.* **2**, 70–75.

Note Added in Proof

Recently, Hammond et al. have shown that ~25 nt RNAs are generated in cultured *Drosophila* S2 cells transfected with *cyclin E* dsRNA (Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. [2000]. *Nature* **404**, 293–296.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☒ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.